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Human anti-smooth muscle antibodies

THESE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Bernard HIRSCHTEL

de

Tramelan-dessus

Thèse N° 3408

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Editions Médecine et Hygiène
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La Faculté de médecine, sur le préavis du professeur Pierre Vassalli et du docteur Giulio Gabbiani, professeur assistant, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

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Le doyen :
William Geisendorf

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I N T R O D U C T I O N

In 1965, Johnson et al. discovered antibodies directed against smooth muscle in the sera of certain patients with liver disease (Johnson, 1965). Since 1971 human anti-smooth muscle sera (HASM) have been used to investigate contractile systems in non-muscular cells.

This thesis will have two parts. The clinical aspects of HASM, i.e. its occurrence in different diseases and its use in diagnosis, are the first to be discussed. In a second chapter HASM will be presented as a laboratory tool to find muscle-related antigen, and results will be described.

PART 1: HASM, CLINICAL ASPECTS.

a) Discovery of antibody to smooth muscle.

In the 1950s autoantibodies were discovered in patients with so-called collagen diseases. In 1963 - 1965 antibodies reactive with nuclei or cytoplasmic components were described in the sera of some patients with liver disease (Bouchier, 1964; Walker, 1965). In 1965, Johnson et al (Johnson, 1965) screened human sera for antibody against gastric parietal-cell cytoplasm. They used an indirect "sandwich" method (see below, page 12) to reveal binding of human sera to rat stomach. Surprisingly they did not only find the staining pattern caused by anti-parietal cell antibody (deBoer, 1965): Some sera produced a pattern which corresponded to the distribution of smooth muscle. Smooth muscle cells between the stomach glands, the muscularis mucosae, the media of the arteries, and the muscle coat of the stomach were colored. This suggested that some human sera contained an immunoglobulin reacting with smooth muscle: human anti-smooth muscle immunoglobulin (HASM). HASM was present in 8 out of 10 sera of patients with probable "lupoid hepatitis", i.e. hepatic cirrhosis accompanied by one or more of the following: characteristic liver biopsy findings, serum anti-nuclear factor, or a coincidental disease of probable autoimmune nature. None of their 25 healthy controls showed HASM (a somewhat surprising finding, see below). Ironside et al. (1966) and Doniach et al. (1966) confirmed Johnson's findings.

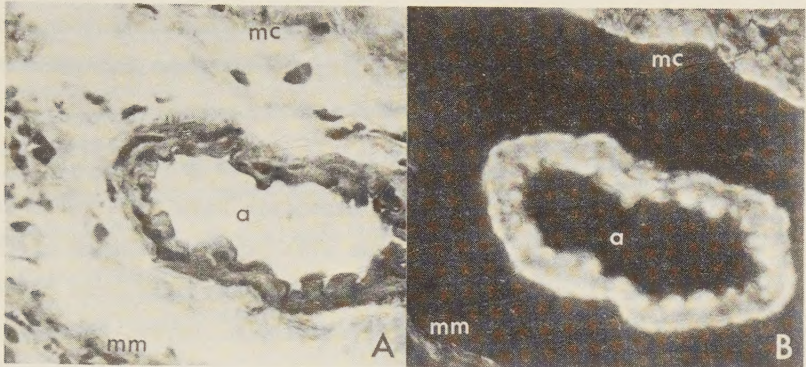


Fig. 1. An arteriole (a) in rat cecum, also showing muscularis mucosae (mm) and muscle coat (mc): (A) stained with hematoxylin-eosin; (B) treated with HASM and stained with fluorescent antihuman IgG; x300.

- b) frequency of occurrence of HASM in different diseases (see also Table 1).

The differential diagnosis of chronic liver disease is difficult. Often, the results of clinical and laboratory examinations will not help to distinguish between chronic persistent and chronic active hepatitis and biliary cirrhosis. Immunological tests are sometimes useful: e.g. the absence of antimitochondrial antibody is incompatible with primary biliary cirrhosis (Doniach, 1966). It is not surprising that many authors have studied the frequency of HASM in different diseases (Johnson, 1965; Doniach, 1966; Farrow, 1970; Vischer, 1970; Holborow, 1972) and the possible use of HASM in diagnosis.

Results of different groups are difficult to compare. Before a serum can be scored "positive" or "negative" for HASM a decision must be made as to the titer that is considered significant. Most authors score all those sera "positive" that stain at a titer of 10 or more. However, Vischer's

"positive" sera all had titers of 40 or more (Vischer, 1970). Not surprisingly, he finds HASM in a much smaller percentage of sera than most others.

There is general agreement that 2/3rd to 3/4th of all patients with active chronic hepatitis have HASM in their sera versus about 1/2 of those with primary biliary cirrhosis. Sera with titers of 100 and above usually come from patients with chronic or aggressive hepatitis (Holborow, 1972), but only a small minority of patients have such high titers. A high titer of HASM supports the diagnosis of chronic active hepatitis and excludes primary biliary cirrhosis, a low titer or the absence of antibody is of no help.

Chronic active hepatitis and primary biliary cirrhosis may have a familiar occurrence. Joske et al. (1970) found five cases of chronic active hepatitis in a single family, and eight other relatives of the same kindred had abnormal immunoglobulin levels. Feizi et al. (1972) reported an increase in mitochondrial antibody among the relatives of 26 patients with primary biliary cirrhosis. Galbraith et al. found HASM in 25 of 33 (75%) patients with chronic active hepatitis, in 8.5% of their relatives, and in 1.9% of age-matched controls. HASM was present in 10 of 22 (45%) of patients with primary biliary cirrhosis, and 11% of their relatives. The difference between relatives and controls was significant at the .01 level (Galbraith, 1974). Most of the relatives with antibodies in their sera had no other signs of liver disease. Raised alkaline phosphatase levels, present in 10% of adult relatives, were not associated with positive tissue antibodies.

HASM has been found in acute infective hepatitis (Farrow, 1970). It was present in 28 of 34 patients during the first four weeks after onset of symptoms. Hepatitis-

associated antigen (HAA) was found in 12 patients; there was no correlation between the presence of HASM and the presence of HAA. Three months or more after onset, HASM persisted in 8 of 21 (40%) patients, hepatitis-associated antigen in only 1. Vischer (1970) investigated 85 patients with chronic hepatitis and found that high titers (40 or more) of HASM and HAA were mutually exclusive. None of the 19 patients with HAA in their sera also had HASM.

Whitehouse et al. report a high incidence of HASM in patients with various cancers: 54 out of 80 patients (70%), and in infectious mononucleosis (80% positive; Holborow, 1971). In the cancer group, SMA was present even when liver metastases could be ruled out. By contrast, in the group with infectious mononucleosis, 90% showed evidence of liver damage (Holborow, 1973).

In conclusion, HASM is present in the sera of a few percent of healthy controls, and in a majority of patients with various diseases, most of which involve the liver. High titers are quite specific for chronic active hepatitis.

c) Pathogenetic significance of HASM.

What role, if any, does HASM have in producing liver lesions? Why should HASM occur so regularly in liver diseases, when smooth muscle fibres are virtually absent in liver tissue? There are yet no definitive answers to these two questions, but experimental studies provide interesting clues.

HASM which gives a pattern of smooth-muscle staining on rat stomach sections also stains the liver cells by outlining them in a polygonal pattern. This suggests that an actomyosin-like protein is present in the liver cell membrane (Holborow, 1972).

T A B L E 1: Frequency of occurrence of HASM.

Author	diagnosis	number of cases	HASM positive	% positive
Johnson, 1965	ACH ¹⁾	10	8	80
Doniach, 1966	ACH	39	26	67
Soloway, 1972	ACH	63	47	75
Serlock, 1973	ACH	not stated		67
Vischer, 1971 ²⁾	ACH	85	11	13
Johnson, 1965	controls ³⁾	25	0	0
Doniach, 1966	controls ⁴⁾	228	3	1
Whitehouse, 1971	controls ³⁾	45	9	20
Vischer, 1971	controls ⁵⁾	58	1	2
Doniach, 1966	PBC	40	20	50
Serlock, 1973	PBC	not stated		50
Vischer, 1971 ²⁾	PBC	13	0	0
Doniach, 1966	IH	12	8	67
Farrow, 1970	IH	39	34	80
Holborow, 1972	mononucleosis	82	65	80
Whitehouse, 1971	various cancers	80	54	67

ACH = active chronic hepatitis; IH = infectious hepatitis; PBC = primary biliary cirrhosis.

Notes: 1) actually "lupoid hepatitis". 2) Positive = antibodies to smooth muscle can be detected after the sera have been diluted 40 times or more. For further explanation see text. 3) healthy hospital personnel. 4) hospital patients with various diseases. 5) patients with liver diseases other than PBC or HCA.

Jones et al. (1970) found that when trypsin-dispersed embryo chick liver cells were exposed to rabbit antiserum raised against human smooth-muscle actomyosin, the spontaneous reaggregation of these cells was prevented. Farrow et al. (1971) prepared monolayers of chick embryo liver cells, and tested sera containing smooth-muscle antibody for their ability to stain these cells. Provided the monolayers had been pretreated by freezing, a characteristic pattern emerged: HASM stained filaments located close to the cell membrane. When HASM was preincubated with human uterine actomyosin the staining was abolished. Fresh, non-frozen cells were not stained.

These experiments indicate that liver cells contain a component cross-reacting with smooth-muscle actomyosin, and that this component is a constituent of a microfilamentous network probably associated with the cell membrane, but not directly accessible at the cell surface, because it needs freezing to be revealed. It is not known how this component is rendered antigenic, but one might speculate that viral infection of liver cells may reveal or alter components immediately beneath the cell surface so that they become immunogenic. In a similar way, neoplastic change might alter cell membranes thus accounting for the surprisingly high incidence of HASM found in various cancers (Holborow, 1972).

HASM can occur in presumably healthy people (Table 1). Therefore it is not surprising that titers of HASM do not correspond to the clinical state in patients with chronic aggressive hepatitis. For instance, in a group of 32 patients with biopsy-proved chronic aggressive hepatitis and treated by prednisone and azathioprine, 23 had HASM initially; 18 lost it during treatment, but in 7 HASM appeared during treatment although their clinical response was good (Soloway, 1972).

PART 2: HASM, A LABORATORY TOOL.

I. INTRODUCTION

a) Fluorescent antibody tracing

Minute amounts of fluorescein are visible when they are illuminated by ultraviolet light. It is possible to conjugate immunoglobulin to fluorescein without altering its capacity to combine with antigen. When fluorescein-conjugated antibody sticks to its corresponding antigen in tissue sections, ultraviolet illumination will reveal the conjugate and thus the antigen.

The tissue section must be prepared in such a way as to avoid damage to the immunological reactivity of the antigen. Frozen sections of unfixed tissues, or of tissues fixed by a few minutes' exposure to ethanol or acetone, are most often used.

The simplest application of the technique is a single treatment with labelled antibody, followed by a wash in physiological buffer-saline to eliminate the excess of labelled uncombined antibody. Such a method has been used for the detection of bacteria, protozoa, and fungal antigens. For every antigen, a specific fluorescein-conjugated antibody must be available. One needs quite a large quantity of strong antisera for conjugation.

However, HASM and other human tissue antibodies are rarely available in huge quantities and high titers. Direct labelling is not feasible, and an indirect (double-layer, sandwich) technique must be used. Sections are first treated with unlabelled human antisera for about 30 minutes. After a thorough wash, labelled anti-human-

globulin is applied; after a second wash, the section is ready for observation in the fluorescence microscope.

Controls must show that any positive staining is specific for a given antigen; for instance, when the first-layer antibody is absorbed with pure antigen, subsequent staining by anti-human-globulin should be negative (Humphrey, 1970).

Antisera for the study of muscle have been used extensively (Finck, 1965); muscle proteins being weak antigens, one must resort to long periods of immunization (Becker, 1969) or to immunization across a wide phylogenetic gap. We did attempt to immunize rabbits against purified rat uterus actomyosin, but without success. The antismooth muscle antibody of patients with chronic active hepatitis provided an easy way out; the sera are available in all large hospitals and maintain their activity for years if stored at -65°C .

b) non-muscular contraction

Muscle is a tissue which contracts. However, contraction is not limited to muscle: An open wound contracts when it closes (Carrel, 1910). Endothelial cells contract when exposed to histamine (Majno, 1969). Chronically ill kidneys and livers contract (Nagle, 1972; Irlé, 1974). Mitral insufficiency may be caused by shortening of chordae tendineae after an attack of rheumatic fever (Scotti, 1971). Platelet contraction is an important step in hemostasis (Lüscher, 1972). Examples could be multiplied almost at will.

The molecular basis of muscular contraction is quite well known. Striated muscle fibers are composed of four major proteins: myosin, actin, tropomyosin, and troponin. Myosin and actin form the muscle filaments observed in the electron microscope. A myosin molecule resembles a thin rod with two small globular heads at one end. Within the thick filaments

the myosin molecules are arranged in a sheaf, forming a cigar-shaped structure. The heads project out of it: they are the minute cross bridges that seem to link the thick and thin filament.

The thin filament is an assembly of actin, tropomyosin, and troponin. Actins are small globular proteins linked to form a double helix like two strands of pearls twisted about each other. Tropomyosin, a long, thin molecule, forms a continuous strand that sits on the strands of actins alongside each groove of the double helix. One tropomyosin extends over seven actin molecules. A globular troponin molecule is attached to the end of each tropomyosin.

During contraction, the interdigitating thick and thin filaments glide upon one another. The myosin heads split ATP to provide energy; they can only do so when they are combined to actin.

Troponin and tropomyosin regulate muscular contraction by interacting with Ca ions. Only when Ca is bound to troponin can myosin heads combine with actin to split ATP. Tropomyosin links troponin and actin; it "tells" the actins it reaches whether Ca is bound to troponin (Murray, 1974).

Thick and thin filaments and all four major muscular proteins exist in smooth muscle (Rüegg, 1971) and in platelets (Lüscher, 1972). Probably, the basis of contraction is much the same as in striated muscle.

All muscle can be easily recognized in the electron microscope. Smooth muscle cells contain microfilaments with a diameter of about 70 Å in an arrangement parallel to the long axis of the cell, nuclear deformations, cytoplasmic dense bodies, and peripheral attachment sites (Bloom, 1968).

Thus, several ways are open to the investigator who sets out to discover muscular features in non-muscular cells. He can

use immunological and chemical methods to find muscular proteins like actin or myosin. By looking at the tissue in the electron microscope he might observe structures characteristic of muscle cells. Last, he may attach strips of tissue to the lever of a kymograph, and record their contraction in response to stimuli which would cause muscle to contract.

c) Experimental models of granulation tissue.

Granulation tissue is the granular newly formed connective tissue in an open wound (Wilhelm, 1971). By extrapolation, any young connective tissue developing close to an area of irritation or injury has been called granulation tissue.

Many experimental models are available:

1. The standardized wound after excision of a square of skin.

In the rat, a wound of 4 cm² will begin to shrink in size about a week later, and will fully close at the end of the second week. Wounds are not ideal for the study of granulation tissue: standardization is difficult, infection quite frequent, and the amount of granulation tissue within them is relatively small (Gabbiani, 1972).

2. In many ways, Selye's granuloma pouch is more practical.

It is produced in the rat by subcutaneous injection of 20 ml of air and 1 ml of 1% croton oil in corn oil. The injected material becomes surrounded by a wall of granulation tissue; the resulting egg-shaped pouch slowly shrinks, starting at about eight days, until it virtually disappears at about three months (Selye, 1953).

3. 2 cm of tail tendon may be homotransplanted into a rat's pelvic fat body; it becomes ensheathed by a layer of granulation tissue which progressively contracts between 7 and 21 days, thus cause a decrease in the overall length of the tendon (Majno, 1958).

4. Blood clots implanted into the rat peritoneal cavity become covered by a few layers of flattened fibroblast-like cells. This newly formed connective tissue does not contain any blood vessels; its contraction cannot be due to vascular smooth muscle (Ryan, 1973).

d) Specificity of HASM

HASM, as its name implies, binds to smooth muscle cells (see above, part I) in frozen cut tissue sections. However, this does not tell us which antigen among the many substances in muscle cells is responsible for the immunological reaction.

HASM also stains platelets. Aggregates of platelets and blood clots contract. This contraction is most probably due to thrombosthenin, an actomyosin-like contractile protein which can be extracted in large amounts from platelets.

Thrombosthenin is composed of thrombosthenin A (actin-like) and thrombosthenin M (myosin-like; Bettex-Galland, 1965).

Gabbiani et al. (1973) used thrombosthenin to establish the specificity of HASM. They incubated HASM with thrombosthenin and purified thrombosthenin A and found that a precipitate formed, and that the serum lost its ability to stain smooth muscle. Incubation with intact platelets or with thrombosthenin M had no such effect, incubation with damaged platelets did abolish some, but not all staining.

These results show that HASM is probably directed against actin, since absorption with highly purified thrombosthenin-A (platelet actin) completely abolishes HASM activity. The experiments with intact platelets exclude the possibility that membranes, which always contaminate thrombosthenin-A to some extent, are responsible for absorption of HASM. Indeed, when HASM is incubated with intact platelets, i.e. placed in contact

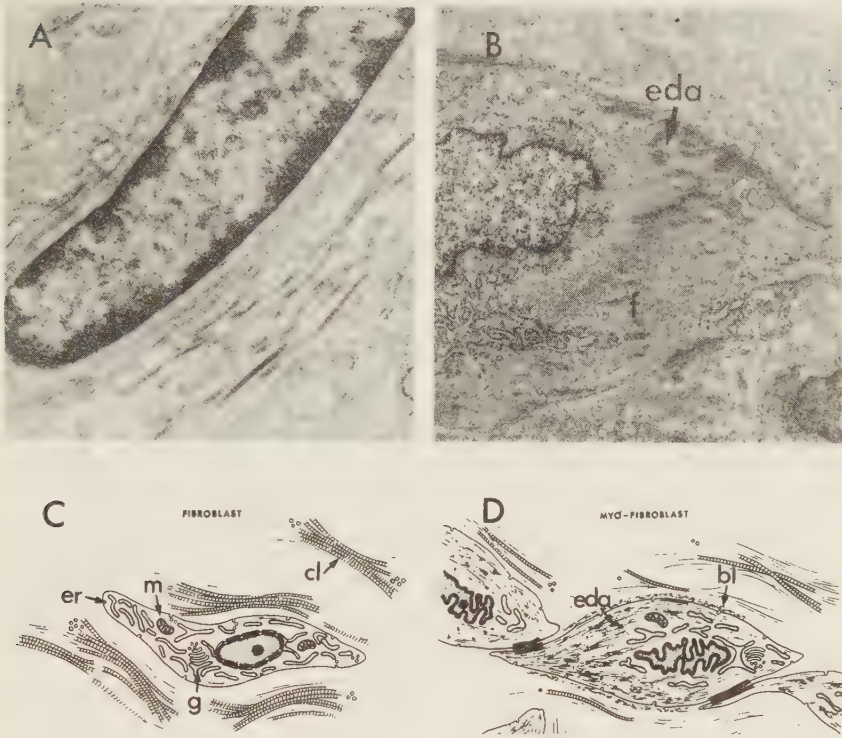


Fig. 2. (A) and (C). Photograph and scheme of a normal fibroblast. The nucleus is smooth. The cytoplasm contains abundant rough endoplasmic reticulum (er), Golgi apparatus (g), mitochondria (m), but only few intracytoplasmic fibrils. The extracellular tissue is mainly composed of collagen buncles (cl).

(B) and (D). Photograph and scheme of a myofibroblast. The nucleus has many folds and indentations. Massive bundles of fibrils (f) fill much of the cytoplasm; electron-dense areas (eda) are scattered among the bundles. Intercellular connections in the form of maculae adhaerentes or desmosomes are present between myo-fibroblasts. Part of the cell surface is covered by a layer of material similar to the basal lamina (bl). The extracellular tissue contains small unbanded fibrils as well as mature collagen fibers (from Gabbiani, G, 1971; and Gabbiani G, Majno G, and Ryan GB: The fibroblast as a contractile cell: The myo-fibroblast. *Biology of the fibroblast*. Edited by E Kulonen and J Pikkarainen, London and New York, Academic Press, 1973, p 139).

only with the platelet membranes, its activity is not affected.

II. EXPERIMENTS WITH HASM

a) granulation tissue (Hirschel, 1971)

It is an established fact that granulation tissue is capable contracting. Abercrombie et al. (1956) have shown that wound contraction occurs in scorbutic animals, when no collagen is formed. This refuted the theory that collagen is responsible for contraction of granulation tissue, and attention has since focused on the fibroblasts (James, 1969). Gabbiani et al. (1971) have demonstrated that fibroblasts of granulation tissue contain a system of intracellular fibrils, "attachment sites", and distorted nuclei typical of contracted cells (see Fig 2). Actomyosin was extracted from Selye's pouches, giving a yield of 4 mg/g of wet weight, and an ATPase activity of 10 nano-moles of ATP split/mg of protein/min - virtually identical values were obtained with similarly prepared extracts from pregnant rat uterus (Majno, 1971).

The term myofibroblast was proposed to designate the modified fibroblasts in contracting granulation tissue. HASM provided further evidence of the similarity between smooth muscle and myo-fibroblasts, i.e., the presence of common antigens in the two types of cells.

Male Wistar rats weighing 100 to 150 g were used. Granuloma pouches were produced according to Selye (1953); and 2 x 2 cm open, full-thickness wounds were made on the skin of the back (Gabbiani, 1971). The rats were killed 7 to 60 days later. HASM serum was obtained from a 16-year-old girl with chronic active hepatitis (courtesy Drs Cruchaud and Nicod, Clinical Immunology Laboratory,

Geneva). Interaction with smooth muscle of rat stomach occurred with dilutions up to 1:320; tests for antimitochondrial and anti-DNA antibodies, and for antinuclear factor, were negative.

To demonstrate smooth muscle antigens, the double layer technique was used (Nairn, 1969). Sections of all tissues were treated with HASM serum at a dilution of 1:6 and stained with sheep antihuman IgG fluorescent serum (Miles-Seravac, Maidenhead, England). Fluorescence was compared with that of sections treated with normal human serum instead of HASM serum. Photographs were taken on a Zeiss UV photomicroscope with UH I or II excitor filter and a Zeiss No. 50/65 barrier filter, using Ektachrome HSB or Ilford HP 4 film. The same fields were photographed with visible light after staining the sections with hematoxylin and eosin.

In the granuloma pouches, labeling was at first recognizable in a few cells on day 7. It reached a maximum between 20 and 30 days, when fluorescent cells were regularly distributed through the whole wall, with the exception of the innermost layer that contained essentially polymorphs and macrophages (see Fig 3). Later, the outer layer of the wall lost its fluorescence: by 50 days, less than half of the wall thickness was labeled.

The open wounds took about 15 days to close; the slides examined were cross sections of the granulation tissue constituting the floor of these wounds. Labeled cells were first detected in 9-day wounds; they were prominent at 11 days, and still present, although less numerous, at 13 and 15 days. The total number of fluorescent cells was always less in wounds than in Selye's pouches. Normal fibroblasts were never stained.

The data show that human antibody against smooth muscle

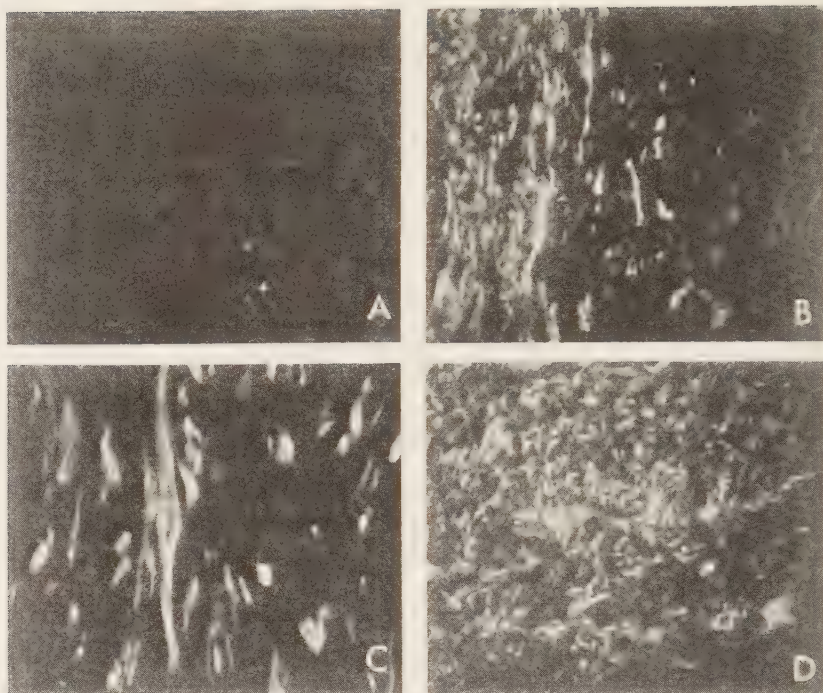


Fig. 3. (A) 28-day-old granuloma pouch, treated with normal human serum followed by fluorescent antihuman IgG; (B) treated with HASM followed by fluorescent antihuman IgG; x 150; (C) treated as in 3B; cytoplasmic contents fluoresce, whereas extracellular material is unstained; x 500; (D) eleven-day-old skin wound, treated as in 3B; x 150.

binds to fibroblasts of rat granulation tissue.

The findings correlate well with earlier data (Gabbiani 1971; Majno, 1971). By electron microscopy, normal fibroblasts contain few or no fibrils; significant bundles are visible in both Selye's pouches and wounds by the time specific fluorescence is detectable. Strips of Selye's pouches contracted in vitro more strongly than strips of wound granulation tissue - and correspondingly more fluorescent cells were found in pouch walls than in wounds.

b) Dupuytren's contracture.

Dupuytren's disease is the progressive, irreversible contraction of one or more digits. The affected structure is the palmar aponeurosis, as described by Dupuytren in 1830; macroscopically, typical nodules are present. These nodules are masses of densely packed cells, probably fibroblasts. The condition is listed among the so-called fibromatoses (Enzinger, 1970) together with the analogous lesion of the plantar aponeurosis named after Ledderhose (Ledderhose, 1897).

In spite of many studies, both etiology and pathogenesis of Dupuytren's disease remain a mystery. Old white men exposed to chronic hand trauma are more frequently affected (Larsen, 1962). Heredity and alcoholism may be other predisposing factors. Because pathologists believed until recently that collagen can shorten in vivo (Payling Wright, 1954), ultrastructural studies of Dupuytren's disease have focused primarily on extracellular material - i.e. on the fibrous part of the fascia. However, no definite abnormality has been found (Dahmen, 1968).

In contrast, Gabbiani and Majno (1972) examined the cells of Dupuytren's nodules. They found a fibrillar system within the cytoplasm, nuclear deformations, and surface differentiations characteristic of smooth muscle and granulation tissue fibroblasts (see preceding section). Not surprisingly, Dupuytren's nodules fluoresced very strongly when treated with HASM and fluorescent anti-human globulin (Montandon, 1973).

It is probable that the "myofibroblasts" are responsible for the shrinkage of the palmar aponeurosis. As cellular contraction is occurring in vivo collagen is also being formed around the cells - this leads to a kind of "catch" (or "lock-step") system: the contraction is irreversible.

c) platelets

Platelet plugs contract in vitro and in vivo (Zucker, 1972). This process is especially obvious in clotted platelet-rich plasma, where the fibrin network and entrapped platelets retract to less than ten percent of the original volume of clotted plasma. Clot retraction probably results from contraction of platelet pseudopods attached to fibrin strands and is due to thrombosthenin, a contractile protein similar to actomyosin (see above and Lüscher, 1972).

Gabbiani et al. (1972) tested interaction of platelets and HASM on two kinds of platelet masses:

1. Platelet pellets. EDTA was added to blood from man or rat. The blood was centrifuged at 77 g; the supernatant was further centrifuged at 4600 g for 30 minutes to produce a pellet of platelets at the bottom of the tube.
2. Experimental thrombi. Rats were injected first with scandium chloride i.v. and thirty minutes later with epinephrine into the thigh muscle. After a day, a thrombus could be harvested from the femoral vein (Gabbiani, 1966).

Unfixed cryostat sections of the platelet pellet of thrombus were then treated as described above for granulation tissue.

No fluorescence was found in sections treated with normal serum. In sections treated with HASM serum heavy fluorescent labeling was present in the centrifuged pellets of human and rat platelets, and in platelet masses found in the thrombi.

Thus, platelets contain smooth muscle antigens. Gabbiani and Ryan (1973) later demonstrated that thrombosthenin-A was responsible for the interaction with HASM (see above).

d) lung

How does the lung adjust perfusion to ventilation and vice versa? Do bronchi and arteries alone regulate blood flow and air supply to the alveoli in response to partial pressure of oxygen and carbon dioxide (Comroe, 1968), or does the alveolus have a role? The lack of substantial musculature in the normal alveolar septa has been used as an argument to rule out any active participation of the alveoli (Fishman, 1963).

Kapanci et al. (Kapanci, 1974) studied alveolar interstitial cells of healthy rats, human, lambs, and monkeys. About 50% of the cells contained bundles of fibrils resembling those found in contractile fibroblasts of granulation tissue and in smooth muscle. When strips of lung tissue devoid of big bronchi and arteries were incubated in an hypoxic atmosphere they contracted, strips cut out of bronchi or arteries relaxed.

The authors used an ingenious method to preserve topography for immunofluorescence. Gelatin was instilled through a tracheal cannula, then the lungs were removed and frozen in liquid nitrogen. Sections were cut in a cryostat, exposed to HASM serum, and stained with fluorescent sheep-anti-human globulin.

There was obvious specific fluorescence within cells in the alveolar walls. Fluorescent cells were scattered in the alveolar septa and around the pre- or postcapillary vessels; there was no fluorescence in peribronchial and perivascular connective tissue.

In conclusion, alveoli contain cells with contractile proteins which resemble the fibroblasts of granulation tissue. Strips containing alveolar tissue respond to hypoxia with contraction,

i.e. in a way which would tend to decrease perfusion. The so-called "contractile interstitial cells" are possible regulators of ventilation/perfusion ratio.

e) kidney contraction

Nagle et al. (1973) studied renal interstitial cells after unilateral ureteral obstruction in rabbits. In the healthy kidney these cells resembled normal fibroblasts of connective tissue; after ureteral obstruction, they developed numerous filaments and dense bodies.

Staining with HASM before obstruction was negative. Eight days after obstruction there was marked increase in labeling of the interstitial cells both in the cortex and in the medulla. At 32 days labeling was still strongly positive.

The authors conclude that renal interstitial cells resemble fibroblasts in their response to injury. Their contraction may be responsible for the shrivelling of an inactive sick kidney.

III. CONCLUSIONS AND PLANS FOR THE FUTURE.

HASM has helped us to find actin in granulation tissue, palmar aponeurosis of Dupuytren's contracture, platelets, normal lung, and chronically ill kidney and liver. These results were obtained by immunofluorescent techniques, i.e. using the light microscope.

Concurrently, we found that all the cells staining with HASM had nuclear deformations, dense bodies, and microfilaments. What structure contains actin and binds HASM? In striated muscle the thin filaments are composed mainly of actin; by analogy it could be argued that in non-muscular contractile cells the microfilaments bind HASM. However, this has not been proved.

Couldn't our studies with HASM be repeated on an ultra-structural level? Might it be possible to see HASM fixed on microfilaments in the electron microscope?

Immunologic techniques have been used in electron microscopy for about 15 years (IEM = immuno-electron-microscopy). Fluorescein is not visible in the electron microscope; other tracers must be combined to antibody. One of these is ferritin, which can be seen directly at high magnification. Another is peroxidase, which forms an electron-dense reaction product after exposure to diaminobenzidine and hydrogen peroxide (Humphrey, 1970). Pictures are of good quality when the antigens to be demonstrated are extracellular: Suspensions of intact cells are exposed to antisera, then to hydrogen peroxide and diaminobenzidine; after centrifugation the pellet is processed for electron microscopy like a conventional tissue block (see for instance Matter, 1972).

Results are less satisfactory for intracellular antigens. The labelled antibody cannot gain access to the antigen unless the cell membrane is disrupted. However, conditions which disrupt the cell membrane (exposure to acetone and alcohol, sectioning of frozen blocks) will destroy ultra-structure to such a degree that electron microscopic pictures are useless. Alternatively, fixation in glutaraldehyde or osmium tetroxide produces excellent electron microscopic photographs, but denatures the antigen and inhibits specific interaction of antigen and antibody (see Avrameas and Miller, 1972, for reviews).

All hope is not lost, however. Using highly purified antibodies, some authors have published pictures of acceptable quality (De Grandi, 1971; Krähenbuehl, 1971 and 1972; Painter, 1973; Leduc, 1969). Most papers describe the distribution of antigens already known by other methods and are content to show that their findings are in agreement.

Much work will have to be done before HASM can be used in electron microscopic studies. The titer is weak and purification by immunoabsorption (Krähenbuehl, 1971) will be a problem. Nobody has yet done EIM on fibroblasts and other non-muscular contractile cells; we will have to find ourselves how to fix and prepare these tissues for IEM.

Once perfected, IEM with HASM will be a powerful means to study the biosynthesis of actomyosin, phagocytosis, the function of lysosomes and phagosomes, the movement of brush borders and ciliated epithelia, contact inhibition and lack thereof in cancer cells, and many other basic cellular processes where contraction and movement are important.

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